

**RUNNING HEAD:**

The rice SOS pathway for salt tolerance.

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**JOURNAL RESEARCH AREA:** Environmental Stress and Adaptation

## CONSERVATION OF THE SOS SALT TOLERANCE PATHWAY IN RICE

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**FOOTNOTES**

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**ABSTRACT**

The salt tolerance of rice (*Oryza sativa*) correlates with the ability to exclude  $\text{Na}^+$  from the shoot and to maintain a low cellular  $\text{Na}^+/\text{K}^+$  ratio. We have identified a rice plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger that, on the basis of genetic and biochemical criteria, is the functional homologue of the *Arabidopsis thaliana* SOS1 protein. The rice transporter, denoted by OsSOS1, demonstrated a capacity for  $\text{Na}^+/\text{H}^+$  exchange in plasma membrane vesicles of yeast cells and reduced their net cellular  $\text{Na}^+$  content. The *Arabidopsis* protein kinase complex SOS2/SOS3, which positively controls the activity of AtSOS1, phosphorylated OsSOS1 and stimulated its activity *in vivo* and *in vitro*. Moreover, OsSOS1 suppressed the salt sensitivity of a *sos1-1* mutant of *Arabidopsis*. These results represent the first molecular and biochemical characterization of a  $\text{Na}^+$  efflux protein from monocots. Putative rice homologues of the *Arabidopsis* protein kinase SOS2 and its  $\text{Ca}^{2+}$ -dependent activator SOS3 were identified also. OsCIPK24 and OsCBL4 acted coordinately to activate OsSOS1 in yeast cells, and they could be exchanged with their *Arabidopsis* counterpart to form heterologous protein kinase modules that activated both OsSOS1 and AtSOS1 and that suppressed the salt sensitivity of *sos2* and *sos3* mutants of *Arabidopsis*. These results demonstrate that the SOS salt tolerance pathway operates in cereals and evidence a high degree of structural conservation among the SOS proteins from dicots and monocots.

## INTRODUCTION

Rice is one of the most important cereal crops in tropical and temperate regions of the world. Among all common environmental stresses, salinity is a major factor decreasing the yield in rice cultivation in coastal areas and in irrigated farmlands. Problems associated with salinity are water deficit imposed by the greater osmolarity of the soil solution and the cellular damage inflicted by excessive ion accumulation in plant tissues. Comparison of rice subspecies and varieties differing in tolerance to salinity has shown that greater tolerance correlates with the ability to exclude  $\text{Na}^+$  from the shoot and maintain a low  $\text{Na}^+/\text{K}^+$  ratio (Gouldack et al., 2003; Lee et al., 2003; Ren et al., 2005). For instance, the salt-sensitive variety IR29 accumulated  $\text{Na}^+$  in leaves at 5- to 10-fold greater concentrations than the salt-tolerant lines BK or Pokkali (Gouldack et al., 2003). In contrast, shoot  $\text{K}^+$  concentration *per se* showed no relation to salinity tolerance in *japonica* spp and only weak correlation in *indica* spp varieties (Lee et al., 2003; Gouldack et al., 2003). Since steady accumulation of  $\text{Na}^+$  is what injures the cells of leaves at moderate salinity levels (Flowers et al., 1991; Munns, 1993), restricting the translocation of  $\text{Na}^+$  is a mechanism for salt tolerance that plays a major role in rice (Lee et al., 2003; Ren et al., 2005). The gene *SCK1/HKT8*, responsible for a major quantitative trait locus (QTL) imparting a high  $\text{K}^+/\text{Na}^+$  balance in shoots and salt tolerance, encodes an  $\text{Na}^+$ -selective transporter of the HKT family that regulates long distance transport of  $\text{Na}^+$  (Ren et al., 2005). *SCK1/HKT8* participate in re-absorption of  $\text{Na}^+$  at the xylem parenchyma, thereby restricting the build up of toxic concentrations of  $\text{Na}^+$  in the photosynthetic tissues (Ren et al., 2005). The related rice gene *HKT1* is preferentially expressed in root xylem parenchyma and in cells adjacent to phloem vessels in leaves, suggesting that it could also be involved in the regulation of long-distance transport of  $\text{Na}^+$  (Gouldack et al., 2002). The mechanism by which rice roots take up  $\text{Na}^+$  is uncertain. Anatomical discontinuities in the root endodermis may lead to uncontrolled apoplastic bypass-flow of ions and their subsequent discharge into the vascular bundle (Yeo et al., 1987; Yadav et al., 1996). In this process, ion transporters would play a minor role or none at all, and the natural variability of salt tolerance among cultivars would be determined by genes controlling developmental traits (Koyama et al., 2001). However, the kinetics of  $\text{Na}^+$  uptake by rice roots is consistent with enzymatic processes driven by ion transporters (Garcia-deblas et al., 2003). Although the molecular identities of these transporters remain to be established, the kinetic properties of OsHKT1 in heterologous systems recapitulate those of whole roots (Garcia-deblas et al., 2003). In wheat, *TaHKT1* is primarily expressed in the root cortex and down-regulation of *TaHKT1* by RNAi reduced  $\text{Na}^+$  uptake and enhanced salt tolerance, indicating that *TaHKT1* mediated  $\text{Na}^+$  uptake (Laurie et al., 2002).

Sodium extrusion at the root/soil interface, as well as some level of  $\text{Na}^+$  efflux in every other cell type to achieve ion homeostasis, is presumed to be of critical importance for the salt tolerance of glycophytes (Tester and Davenport, 2003). Indeed, efficient efflux of  $\text{Na}^+$  to the soil solution must function in the roots of several species to minimize net uptake since unidirectional influx of

Na<sup>+</sup> is rapid and greatly exceeds the rate of accumulation (Tester and Davenport, 2003). In wheat roots, high rates of Na<sup>+</sup> efflux were inferred because net uptake was very low relative to unidirectional influx (Davenport et al., 2005). The Na<sup>+</sup>/H<sup>+</sup> antiporter SOS1 is the only Na<sup>+</sup> efflux protein at the plasma membrane of plants characterized so far. Mutants of *Arabidopsis thaliana* lacking SOS1 are extremely salt sensitive and have combined defects in Na<sup>+</sup> extrusion and in the long-distance transport of this ion from root to shoot (Qiu et al., 2002; Shi et al., 2002). SOS1 is primarily expressed at the root tip epidermis and in xylem parenchyma at the xylem/symplast boundary throughout the plant (Shi et al., 2002). At the root/soil interface, SOS1 would act extruding the excess of Na<sup>+</sup> ions from root epidermal cells. In addition, the analysis of the Na<sup>+</sup> root/shoot partition in the *sos1* mutant under different saline regimes indicated that SOS1 also participated in the redistribution of Na<sup>+</sup> between roots and shoot in a complex manner (Shi et al. 2002). Under moderate saline stress (25 mM NaCl) *sos1* mutant plants accumulated less Na<sup>+</sup> in their aerial parts than the wild-type, indicating that SOS1 functions in loading Na<sup>+</sup> into the xylem for controlled delivery to the shoot. By contrast, at high salinity (100 mM NaCl), the roots and aerial parts of *sos1* mutant plants accumulated more Na<sup>+</sup> than wild-type plants, which could be caused by the breakdown of Na<sup>+</sup> exclusion at the root epidermis and to the large electrochemical gradient of Na<sup>+</sup> across the xylem/symplast boundary (Pardo et al., 2006; Shi et al. 2002). In addition, it has been suggested that under severe salinity stress the difference of Na<sup>+</sup> concentration between the xylem sap and xylem parenchyma cells could be of greater magnitude than the corresponding pH gradient, which would result in reversal of SOS1 activity (assuming an electroneutral exchange) and retrieval of Na<sup>+</sup> from the xylem (Shi et al., 2002; Tester and Davenport 2003). The activity of the SOS1 exchanger is regulated through protein phosphorylation by the SOS2/SOS3 kinase complex (Quintero et al. 2002; Qiu et al., 2002). SOS2 is a serine/threonine protein kinase belonging to the SnRK3 family (Gong et al. 2004; Kolukisaoglu et al. 2004). SOS3 is a myristoylated Ca<sup>2+</sup> sensor belonging to the recoverin-like family of SCaBPs/CBLs (Gong et al. 2004; Kolukisaoglu et al. 2004). Upon Ca<sup>2+</sup> binding, SOS3 undergoes dimerization and enhances the protein kinase activity of SOS2 (Guo et al. 2001; Sánchez-Barrena et al. 2005). Besides activating SOS2, SOS3 was also shown to recruit SOS2 to the plasma membrane to achieve efficient interaction with SOS1 (Quintero et al. 2002). Mutant plants deficient in either SOS2 or SOS3 share the salt-sensitive phenotype of *sos1* plants (Zhu, 2000).

We have begun to characterize Na<sup>+</sup> efflux proteins of rice by isolating a SOS1 homologue, which is encoded by a single copy gene. We show that OsSOS1 functions as a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter in yeast cells and that, like its *Arabidopsis* counterpart, it is phosphorylated and activated by the SOS2/SOS3 protein kinase complex. Ectopic expression of OsSOS1 suppressed the growth defects of an *Arabidopsis sos1* mutant line. We have also identified the homologues of AtSOS2 and AtSOS3 (OsCIPK24 and OsCBL4, respectively) which coordinately regulate the activity of OsSOS1. These results show that the SOS pathway for salt tolerance operates in cereals.

## RESULTS

### Cloning of OsSOS1 and sequence analyses.

A homology-based computer search identified a rice 290-bp EST clone (*Oryza sativa* subsp. *japonica* cv *Nipponbare*; Accession C71771) encoding a putative homologue of the *Arabidopsis thaliana* Na<sup>+</sup>/H<sup>+</sup> antiporter SOS1. The EST clone was obtained from the MAFF DNA Bank (Japan) and the entire cDNA insert was sequenced. Sequence comparison with the *Arabidopsis* SOS1 protein indicated that the rice EST clone encoded a full-length SOS1 homologue, although the ORF was presumably interrupted by 2 unspliced introns. These conclusions were supported by sequence comparisons that were extended to include additional SOS1 homologues from other plant species. Sequences of SOS1 homologues from the moss *Physcomitrella patens* and the seagrass *Cymodocea nodosa* are available from public databases (Accessions CAD91921 and CAD20320, respectively). Sequences from the *Arabidopsis* relative *Thellungiella halophila* and from the facultative halophyte *Mesembryanthemum crystallinum* were kindly provided by Valery Poroyko and Hans Bohnert (University of Illinois). Multiple sequence alignments of these polypeptides (data not shown) indicated a high degree of amino acid sequence co-linearity among SOS1 homologues and evidenced the presence of 2 intervening nucleotide sequences of 805 and 160 bp in the rice EST clone at positions +750 and +970, respectively (numbering relative to the ATG codon in the spliced rice cDNA). That these intervening sequences were in fact unprocessed introns was confirmed by the analysis of the rice genome sequence at the corresponding locus. To obtain a fully spliced cDNA that could be used for functional studies, RT-PCR amplification was performed with rice RNA as template and oligonucleotides that primed the amplification of a fragment spanning the region that contained the 2 unprocessed introns in the EST clone. The amplified fragment was sequenced to confirm the removal of all introns and the fidelity of the polymerase and was subsequently subcloned into the EST clone replacing the unspliced portion of the cDNA. In this way, a full-length cDNA was created that encoded a 1148 amino acid protein with 57.5% similarity to the *Arabidopsis* SOS1 transporter (Supplemental Figure 1). A search of the available rice genomic sequences (ssp. *indica* and *japonica*) indicated that the isolated OsSOS1 cDNA corresponded to locus Os12g44360 on chromosome XII (25285 - 35082 bp), with no additional sequences of significant similarity. Therefore, SOS1 is a single copy gene in rice.

### The sodium transport activity of OsSOS1 is stimulated by the *Arabidopsis* SOS2/SOS3 kinase complex

Cells of the *Saccharomyces cerevisiae* strain AXT3K ( $\Delta ena1-4 \Delta nha1 \Delta nhx1$ ) lack all major sodium transporters essential for the sodium tolerance of yeast and thus are incapable of

growing in AP medium with Na<sup>+</sup> concentrations higher than 70 mM (in 1 mM K<sup>+</sup>) (Quintero et al., 2002). The P-type pumps ENA1-ENA4 and the (Na<sup>+</sup>,K<sup>+</sup>)/H<sup>+</sup> antiporter NHA1 localize to the plasma membrane and both mediate Na<sup>+</sup> efflux (Wieland et al., 1995; Bañuelos et al., 1998), whereas the Na<sup>+</sup>/H<sup>+</sup> antiporter NHX1 drives Na<sup>+</sup> sequestration into endosomal compartments (Darley et al., 2000; Quintero et al., 2000). When OsSOS1 was expressed in AXT3K cells from a multicopy vector (pDR195), halotolerance was partially recovered and cells could grow in AP medium containing up to 100 mM sodium (1 mM K<sup>+</sup>) (Figure 1A).

The *Arabidopsis* AtSOS1 transporter has been shown to be phosphorylated and activated by the SOS2/SOS3 protein kinase complex (Qiu et al., 2002; Quintero et al., 2002). SOS2 is a Ser/Thr protein kinase whose activity and subcellular localization are dependent on the interaction with the calcium sensor protein SOS3 (Halfter et al., 2000; Quintero et al., 2002). To test whether the rice homologue OsSOS1 could also be activated by the *Arabidopsis* SOS2/SOS3 kinase complex, *A. thaliana* SOS2 and SOS3 proteins were co-expressed in AXT3K yeast along with OsSOS1, and the effect of these combinations on the halotolerance of transformants was determined. As shown in Figure 1A, the concurrent expression of the three SOS proteins greatly increased the salt tolerance of the transformed cells and afforded growth in media with up to 400 mM sodium, which is near the maximal salt concentration tolerated by wild-type yeast cells in AP medium (data not shown). This enhanced tolerance was observed only when OsSOS1 was present, negating that SOS2 and SOS3 were unmasking an endogenous yeast activity. AtSOS2 alone in the presence of OsSOS1 was capable of enhancing the salt tolerance of yeast to an intermediate level lower than that under co-expression of the three SOS proteins but still considerably higher than that with OsSOS1 alone. Presumably, this enhanced salt tolerance resulted from the basal activity of SOS2 in the absence of SOS3.

To determine how OsSOS1 conferred greater Na<sup>+</sup> tolerance to yeast, we measured the Na<sup>+</sup> content of cells expressing various combinations of SOS proteins. Cells expressing OsSOS1 alone and growing in AP with 30 mM NaCl (1 mM KCl) maintained their intracellular sodium levels considerably lower than did control cells transformed with an empty vector (Figure 1B). The intracellular Na<sup>+</sup> content was minimal when the three SOS proteins were co-expressed, well below that of cells expressing OsSOS1 alone or together with SOS2. Thus, the association between the halotolerance and cytoplasmic sodium levels suggests that OsSOS1 functions as a sodium efflux transporter in yeast and that its activity is greatly enhanced by the SOS2/SOS3 kinase complex. To directly demonstrate Na<sup>+</sup>/H<sup>+</sup> antiporter activity of OsSOS1, plasma membrane vesicles were purified by use of aqueous two-phase partitioning from yeast expressing OsSOS1 with and without co-expression of the SOS2/SOS3 kinase complex. Cells were grown on selective AP medium containing 1 mM KCl and transferred to the same medium supplemented with 100 mM NaCl for 1 hour to ensure activation of the SOS2/SOS3 kinase complex when present. The purity of vesicle preparations was tested by measuring ATP



hydrolysis in the presence of inhibitors of mitochondrial (azide), vacuolar (nitrate) and plasmalemma (vanadate) ATPases. The relative sensitivity of total ATPase activity to these inhibitors demonstrated that vesicle preparations were enriched in plasma membrane (data not shown).  $\text{Na}^+/\text{H}^+$  exchange was monitored by the quinacrine fluorescence quenching method. An inside-acid proton gradient ( $\Delta\text{pH}$ ) across vesicle membranes was established after the addition of ATP (Figure 2A). Once  $\Delta\text{pH}$  reached a steady state, the addition of sodium salts led to fluorescence recovery (i.e. dissipation of the  $\Delta\text{pH}$ , Figure 2A). Vesicles isolated from yeast cells expressing OsSOS1 displayed  $\text{Na}^+/\text{H}^+$  exchange activity that was greater than background exchange in AXT3K recipient cells over 25-200 mM  $\text{Na}_2\text{SO}_4$  (Figure 2B). Maximal  $\text{Na}^+/\text{H}^+$  exchange activity, which was detectable at lower  $\text{Na}^+$  concentrations, was observed in cells co-expressing OsSOS1 and the *Arabidopsis* SOS2/SOS3 kinase complex, as was expected from activation of the rice  $\text{Na}^+$  transporter by the SOS2/SOS3 kinase. These differences in  $\text{Na}^+/\text{H}^+$  exchange activity are in agreement with the relative tolerance to NaCl and intracellular  $\text{Na}^+$  content of these transformants (see Figure 2). To further ensure that  $\Delta\text{pH}$  dissipation was due to  $\text{Na}^+/\text{H}^+$  exchange, transport assays were performed with sodium gluconate as a substrate. Relative  $\text{Na}^+/\text{H}^+$  exchange rates among vesicle preparations were commensurate at identical  $\text{Na}^+$  concentrations independently of the salt used (Figure 2C). Since gluconate is an impermeant anion, unlike sulfate,  $\Delta\text{pH}$  dissipation was concluded to be specific for  $\text{Na}^+$ . Transport assays in the presence of  $\text{K}^+$  and valinomycin, to dissipate the electrical membrane potential, produced similar results (data not shown), further indicating that  $\text{Na}^+$  did not move electrophoretically and that the  $\text{Na}^+/\text{H}^+$  exchange mediated by SOS1 was electroneutral. An approximate  $K_m$  of 29 mM for  $\text{Na}^+$  was estimated for activated OsSOS1 in the range of 6.5 – 100 mM  $\text{Na}^+$  after subtracting background transport in control vesicles from the AXT3K strain.

We have previously shown that the *Arabidopsis* SOS1 protein is a substrate for the SOS2/SOS3 kinase complex (Quintero et al., 2002). Results depicted in Figures 1 and 2 indicate that the activity of the rice SOS1 transporter is also enhanced by the co-expression of the *Arabidopsis* protein kinase. To confirm that OsSOS1 was phosphorylated by AtSOS2, C-terminal histidine-tagged OsSOS1 and AtSOS2 were purified from yeast membranes by  $\text{Ni}^{2+}$ -binding chromatography (Figure 3A). Also a mutant form of SOS2 (SOS2T/D $\Delta$ 308) that is hyperactive and independent of SOS3 *in vitro* (Guo et al., 2001) was purified as a translational GST fusion (Figure 3A). As shown in Figure 3B, OsSOS1 was weakly phosphorylated by the wild-type SOS2 (in the absence of SOS3) compared to the strong phosphorylation catalyzed by the recombinant SOS2T/D $\Delta$ 308 kinase, thus demonstrating that the rice protein is recognized as a legitimate substrate by the *Arabidopsis* protein kinase.

#### Complementation of *Arabidopsis* *sos1* mutant

In *Arabidopsis*, AtSOS1 has been shown to fulfill two important roles pertaining to sodium tolerance, namely the restriction of net  $\text{Na}^+$  uptake by roots and the control of xylem loading for

long-distance transport (Shi et al., 2002). To extend the functional comparisons between OsSOS1 and AtSOS1 to whole plants, a full-length cDNA encoding the rice transporter was transformed into the *Arabidopsis* mutant *sos1-1*, which bears a 14-bp gene deletion that completely abrogates AtSOS1 activity (Shi et al., 2000; Qiu et al., 2002). Transgenic seedlings (T<sub>1</sub> generation) were selected on MS medium with kanamycin and then transferred to MS supplemented with 50 mM NaCl to test genetic complementation (Figure 4). Ten out of 16 transgenic T<sub>1</sub> seedlings tested continued growing in the presence of salt (Figure 4A) and were successfully transferred to soil for further analyses. In all these 10 complemented lines, expression of the *OsSOS1* transgene was confirmed by RT-PCR with the use of rice-specific primers, and the *sos1-1* genetic background was verified by diagnostic PCR of the 14-bp deletion that defines this mutant allele (data not shown) (Shi et al., 2000). Mutant *sos1-1* plants showed a moderate growth defect in MS plates, which was further exacerbated by the imposition of moderate salinity stress (Figure 4B). Phenotypic suppression of the *Arabidopsis* *sos1-1* mutant by the rice *SOS1* cDNA was partial because of the greater growth of congenic lines transformed with the *Arabidopsis* *SOS1* cDNA as compared with transgenic lines expressing the rice protein, both in the absence and presence of NaCl (Figure 4B). These results indicate that the rice *SOS1* protein can substitute for the endogenous transporter of *Arabidopsis*, albeit not completely.

#### **OsSOS1 transcript levels in response to salt stress.**

To learn whether the expression of *OsSOS1* is modified under salt stress, a northern blot membrane was prepared with total RNA purified from root and shoot of rice plants subjected to 100 mM NaCl salinity stress for 3, 15 and 48 h. Hybridization was performed with a probe corresponding to the C-terminal part of *OsSOS1*, and radiometric signals were quantified by densitometric scanning of autoradiograms. The relative signal intensities were normalized by re-probing the blots with an 18S rRNA gene probe. Basal *OsSOS1* expression levels were detected in root and shoot of control, non-treated plants (Figure 5). After 3-h salt treatment, roots showed a slow and transient increase in the level of *OsSOS1* transcripts, which reached a maximal 6-fold induction 15 h after the onset of salt stress (Figure 5). By contrast, the effect of salt stress in shoots was the opposite. After 3-h salt treatment, the level of *OsSOS1* transcripts decreased, reaching a 5-fold reduction in mRNA abundance relative to basal levels in control plants (Figure 5). This decrease was transient and the mRNA abundance slowly recovered, re-establishing near-basal levels after 48 h of salt treatment. These results further suggest a functional role of *OsSOS1* in the response of rice plants to salt stress. The changes in mRNA abundance in roots over the first day after transfer to salt were likely related to the osmotic shock rather than to ionic stress, whereas the up-regulation in roots and leaves at later times after turgor recovery (48 h) was the likely result of sodic stress.

### Identification of a rice kinase complex that activates OsSOS1

Analysis of the rice genome allowed for the identification of up to 30 CIPK/PKS kinases of the SnRK3 family and 10 CBL/SCaBP interacting calcium sensors (Kolukisaoglu et al., 2004). On the basis of amino acid sequence comparisons, the rice proteins most similar to the *Arabidopsis* SOS3 protein are OsCBL4 (Os05g45810), OsCBL7 (Os02g18880) and OsCBL8 (Os02g18930), with 66.2%, 67.1% and 66.7% identity to AtSOS3, respectively (Supplemental Figure 2). Like AtSOS3, all three rice homologues are predicted to be N-myristoylated proteins, a post-translational modification that is essential for SOS3 functionality (Ishitani et al., 2000). The remaining OsCBL proteins show significantly low similarity scores (Hwang et al., 2005). All CBL proteins contain 4 conserved EF-hand motifs for  $\text{Ca}^{2+}$  binding, separated by intervening sequences of fixed length. Size and sequence variations among CBL proteins are therefore restricted largely to N- and C-terminal extension from the conserved core (Kolukisaoglu et al., 2004). Because the N-terminal part of AtSOS3 is more similar to that of OsCBL4 than OsCBL7 and OsCBL8 (Supplemental Figure 2) and OsCBL4 localizes to the plasma membrane as does SOS3 (Hwang et al., 2005), a full-length cDNA of OsCBL4 was amplified by RT-PCR with total mRNA isolated from *O. sativa* cv Nipponbare used as a template as described in Methods. To test its putative functional identity with AtSOS3, OsCBL4 was cloned in plasmid pDR195 to express the rice protein from the *PMA1* gene promoter. The resulting plasmid, pDROsCBL4, was transformed in the yeast strain YP890, expressing AtSOS1 from a chromosomal integration, with or without AtSOS2 as the interacting protein kinase. As shown in Figure 6A, OsCBL4 was competent for interaction with AtSOS2 and activation of AtSOS1, as expected if OsCBL4 were a functional homologue of AtSOS3. Moreover, expression of OsCBL4 suppressed completely the salt sensitivity of the *Arabidopsis* mutant *sos3-1* (Figure 7A, C). The *sos3-1* mutant bears a 9 bp deletion starting at position 759 from the translation start codon (Liu and Zhu, 1998). Five *sos3-1* lines ( $T_2$  plants) showing genetic complementation were further analyzed; and all tested positive for expression of OsCBL4 on RT-PCR. They also tested positive for the *sos3-1* genetic background on diagnostic PCR for the signature 9-bp deletion (results not shown). Together, these data indicate that OsCBL4 can functionally substitute for AtSOS3.

The *Arabidopsis* protein kinase SOS2/CIPK24 is most similar to the uncharacterized rice proteins OsCIPK24 (Os06g40370; 68.2% identity) and OsCIPK8 (protein accession BAD87720; 62% identity). Because OsCIPK8 is also more similar to *Arabidopsis* AtCIPK8 than to AtSOS2 (Kolukisaoglu et al., 2004), we focused our initial analysis on OsCIPK24. A full-length cDNA was amplified by RT-PCR with total mRNA isolated from *O. sativa* cv Nipponbare used as a template as described in Methods. Oligonucleotides for amplification were designed according to the available sequence of a potential full-length cDNA sequence of OsCIPK24 (database entry AK102270) coding for a putative 454-amino acid polypeptide. The cDNA was cloned in the yeast vector p414GPD and transformed in the yeast strain YP890 (*PGK1::AtSOS1::CYC1*), with

and without the potentially interacting protein OsCBL4. Unexpectedly, OsCIPK24 failed to activate AtSOS1 in the yeast system (results not shown). To test whether this failure was due to the inability of OsCIPK24 to interact with OsCBL4 or to recognize AtSOS1 as a substrate, a constitutive CBL-independent form of OsCIPK24 was produced by deletion of the conserved C-terminal autoinhibitory domain present in SnRK3 kinases (Albrecht et al., 2001; Guo et al., 2001). On the basis of the known localization of the autoinhibitory domain of AtSOS2 (Guo et al., 2001), a C-terminal truncation of OsCIPK24 was produced by introducing a stop codon at the leucine residue at position 315 (Supplemental Figure 3). The truncated protein was tested for its ability to activate AtSOS1 or the rice protein OsSOS1, with negative results in both cases (data not shown), which suggests that the OsCIPK24 was not a functional homologue of AtSOS2 or that the protein being produced in yeast was not biologically active. Close inspection of the predicted open reading frame of the cDNA amplified based on the EST database entry AK102270 revealed that a conceptual translation starting at the downstream ATG codon corresponding to methionine in position +7 predicted a shorter protein with greater similarity to the amino terminal part of AtSOS2 than the one produced by the larger ORF present in the OsCIPK24 cDNA amplified initially (Supplemental Figure 3). Hence, 7-amino acid shorter versions of OsCIPK24, with or without the C-terminal deletion at the leucine residue at position 309 (numbering relative to the new start codon; denoted OsCIPK24 $\Delta$ 309), were cloned in plasmid p414GPD and tested in yeast cells. As depicted in Figure 6B, the constitutive protein kinase OsCIPK24 $\Delta$ 309 was able to activate AtSOS1 in the absence of OsCBL4, which demonstrates that the polypeptide with the shortened N-terminal was biologically active. Moreover, co-expression of the complete OsCIPK24 (i.e., starting at the second methionine but with the intact C-terminal inhibitory domain) interacted productively with OsCBL4 to activate the SOS1 exchangers from both *Arabidopsis* (AtSOS1) and rice (OsSOS1) but failed to fully activate these exchangers in the absence of OsCBL4 (Figure 6C, D). Finally, the OsCIPK24 kinase (short N-terminal) was also able to suppress the salt sensitivity of an *Arabidopsis* *sos2-2* mutant completely (Figure 7B, D). The *sos2-2* allele contains a 2-bp deletion corresponding to nucleotides 1521 and 1522 relative to the translational start codon of SOS2 (Liu et al. 2000). Six transgenic lines ( $T_2$  plants) showing genetic complementation for salt tolerance tested positive for expression of the *OsCIPK24* transgene on RT-PCR and for the signature deletion of the *sos2-2* mutant allele on diagnostic PCR (results not shown). Together, these results strongly suggest that OsCIPK24 is the functional rice homologue of the *Arabidopsis* SOS2. They also show that the OsCIPK24/OsCBL4 complex form a functional module with OsSOS1 to achieve sodium tolerance.

## DISCUSSION

Our genetic and biochemical analyses show that a rice protein, OsSOS1, with significant sequence similarity to the SOS1 sodium exchanger from *A. thaliana*, is also its functional homologue. First, OsSOS1 suppressed the Na<sup>+</sup> sensitivity of a yeast mutant lacking the major

Na<sup>+</sup> efflux systems at the plasma membrane (the array of ENA1-ENA4 Na<sup>+</sup> pumps and the (Na<sup>+</sup>,K<sup>+</sup>)/H<sup>+</sup> exchanger NHA1) by a mechanism that reduced the net cellular Na<sup>+</sup> content (Figure 1). Furthermore, plasma membrane preparations from yeast transformants expressing OsSOS1 demonstrated greater capacity for Na<sup>+</sup>/H<sup>+</sup> exchange (Figure 2). Second, the *Arabidopsis* protein kinase complex SOS2/SOS3, which positively controls the activity of AtSOS1 (Qiu et al., 2002; Quintero et al., 2002), also stimulated the activity of OsSOS1, both *in vivo* (Figure 1) and in plasma membrane vesicles (Figure 2B and C), and OsSOS1 was recognized as a genuine phosphorylation substrate by AtSOS2 (Figure 3B). Third, OsSOS1 suppressed the growth defect, both in the absence and presence of salt, of a *sos1-1* mutant of *A. thaliana* (Figure 4). Taken together, these results demonstrate that OsSOS1 is a functional homologue of AtSOS1, and they represent the first molecular and biochemical characterization of a Na<sup>+</sup> efflux protein from monocots. Rice OsSOS1 catalyzed Na<sup>+</sup>/H<sup>+</sup> exchange in plasma membrane vesicles derived from the yeast strain AXT3K in which genes encoding the Na<sup>+</sup> efflux proteins ENA1-ENA4 and NHA1 had been deleted. Although these proteins account for most of the Na<sup>+</sup> efflux in yeast plasma membranes and the cellular tolerance to Na<sup>+</sup> (Bañuelos et al., 1998), some capacity for Na<sup>+</sup>/H<sup>+</sup> exchange still remained in plasma membrane vesicles of the strain AXT3K at Na<sup>+</sup> concentrations greater than 50 mM (Figure 2B). Expression of OsSOS1 alone imparted a moderate enhancement of Na<sup>+</sup>/H<sup>+</sup> exchange above that background activity. In contrast, co-expression of the rice SOS1 with the activating SOS2/SOS3 complex from *Arabidopsis* significantly increased the Na<sup>+</sup>/H<sup>+</sup> exchange in vesicles. An approximate K<sub>m</sub> of 29 mM for Na<sup>+</sup> was estimated for activated OsSOS1, which is similar to the K<sub>m</sub> of 23 mM Na<sup>+</sup> that has been estimated for AtSOS1 in plasma membrane vesicles of *Arabidopsis* plants subjected to salt exposure to induce the activity of AtSOS1 (Qiu et al., 2003). Accordingly, the salt tolerances imparted to yeast cells by OsSOS1 and AtSOS1 were comparable (Figure 6A and D).

The rice OsSOS1 protein was phosphorylated and activated by the *Arabidopsis* SOS2/SOS3 proteins (Figures 2 and 3), which strongly suggests that the mechanistic details of the biochemical regulation of SOS1 proteins are conserved among these species and that, as a consequence, functional homologues of the SOS2 and SOS3 proteins should exist in rice. On the basis of protein sequence similarity, functional tests in yeast, and genetic complementation of *Arabidopsis* mutants, we have isolated likely candidates as the functional rice homologues of the SOS2/SOS3 kinase complex. The rice proteins OsCIPK24 and OsCBL4 were able to activate the rice transporter OsSOS1 and they could also be exchanged with their *Arabidopsis* counterparts to form heterologous protein kinase modules that were fully competent to activate the SOS1 Na<sup>+</sup>/H<sup>+</sup> antiporters from both *Arabidopsis* and rice in yeast cells (Figures 1 and 6). Their ability to complement the *sos2* and *sos3* mutations of *Arabidopsis* imply that they are able to form heterologous protein kinase complexes *in planta* also (Figure 7). We have previously shown that over-expression of SOS2 or SOS3 failed to increase the salt tolerance of complemented mutant lines above wild-type levels (Guo et al., 2004), which is consistent with the data presented in Figure 7 showing that the over-expression of the rice proteins brought the

salt tolerance of the *Arabidopsis* mutants close but not beyond that of wild-type plants. These results show a high degree of structural conservation among the SOS proteins from dicots and monocots. Contrary to *OsSOS1*, which is a single-copy gene in rice, *OsCIPK24* and *OsCBL4* belong to a gene family. Although the complete genomes of the subspecies *japonica* and *indica* are assembled only in part, in both cases the same set of 10 CBLs and 30 CIPKs have been identified *in silico* (Kolukisaoglu et al., 2004). Hence, it remains a possibility that other rice CIPK and CBL isoforms could also have tested positive in our functional analyses, especially for *OsCBL4*, for which there were at least 2 other candidates, *OsCBL7* and *OsCBL8*, with high sequence similarity and predicted N-terminal myristoylation (Kolukisaoglu et al., 2004; Hwang et al., 2005). In rice, the predicted polypeptides of *OsCBL1*, 4, 5, 7 and 8 are potential substrates for N-myristoylation, a lipid modification that promotes protein-protein or protein-membrane interactions in eukaryotic cells (Kolukisaoglu et al., 2004). However, only *OsCBL4* has been localized to the plasma membrane (Hwang et al., 2005), which is consistent with a role in *SOS1* activation (Quintero et al., 2002). Nonetheless, since CIPK/PKS protein kinases can interact with more than one CBL/SCaBP protein (Guo et al., 2001; Kolukisaoglu et al., 2004), whether *OsCBL4*, *OsCBL7* and/or *OsCBL8* are functionally redundant needs to be investigated. The phenotypic analysis of the growing number of annotated insertional mutants in rice should enable the unequivocal identification, by genetic criteria, of the isoforms of CBLs and CIPKs that are relevant for salt tolerance.

Root hairs and epidermal cells are primary sites for controlled uptake of inorganic ions that enter the symplastic pathway. In addition, ions move apoplastically across the cortex with the bulk flow of water and solutes until they reach the endodermis barrier that prevents further diffusion into the vasculature and entails the selective uptake of ions into the symplast. In a saline environment, the cell-to-cell pathway imparts greater selectivity and reduced ion uptake than the bulk flow of water and solutes along the root apoplastic pathway. The relative contribution of each pathway to net flow of water and solutes is affected by environmental factors, including salinity (Steudle and Peterson, 1998; De Boer and Volkov, 2003). Typically, under stress conditions, the apoplastic pathway is reduced by a process that involves the lignosuberization of root tissues (Azaizeh and Steudle, 1991; Cruz et al, 1992; Sanchez-Aguayo et al., 2004). Notwithstanding this general model, sodium uptake has been suggested to occur in rice roots primarily via apoplastic bypass-flow and leakage into the xylem (Yeo et al., 1987). This apoplastic pathway is presumed to involve discontinuities along the rhizodermal and endodermal barriers (Yeo et al., 1987; Yadav et al., 1996). Consequently, the uptake of sodium in rice in saline conditions was proposed to be controlled by genes affecting root anatomy rather than membrane transport processes (Koyama et al., 2001). However, even if uncontrolled apoplastic flow were the predominant pathway for  $\text{Na}^+$  entry the root vascular cylinder, an enzymatic  $\text{Na}^+$  efflux mechanism would still be of critical importance to minimize cellular  $\text{Na}^+$  accumulation in tissues along the plant axis. The plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter *SOS1* could likely fulfill this role and prevent cellular injury, thus contributing to whole plant salt

tolerance, particularly at moderate salinity levels that do not cause catastrophic physiological failure. Low salt concentration may not, in itself, be damaging to rice (Yeo et al., 1991); it is the progressive increase in internal concentration that leads to cellular damage and growth impairment (Munns, 1993). As long as growth is sustained to keep the concentration of salt in leaf tissues low by dilution, significant damage could be averted, since the long-term build-up of salt in leaves is what ultimately leads to injury (Flowers et al., 1991). Although enhanced ion compartmentation in vacuoles may increase the salt tolerance of rice (Ohta et al., 2002; Fukuda et al., 2004), this species is a typical glycophyte that relies primarily on  $\text{Na}^+$  exclusion for growth in saline environments (Golldack et al., 2003; Lee et al., 2003).

In support of the critical involvement of ion transport processes in the  $\text{Na}^+$  accumulation by rice roots, the ability of salt-tolerant lines to exclude  $\text{Na}^+$  depends on the  $\text{K}^+$  concentration in the growth medium. At high- $\mu\text{M}$  to low- $\text{mM}$   $\text{K}^+$  concentrations, the salt-tolerant lines Pokkali and BK selectively excluded  $\text{Na}^+$  while keeping the internal  $\text{K}^+$  concentration fairly constant. In contrast, at low- $\mu\text{M}$   $\text{K}^+$ , these lines accumulated  $\text{Na}^+$  similar to the salt-sensitive line IR29 (Golldack et al., 2002; Golldack et al. 2003). Physiological measurements of ion uptake by rice roots indicate the existence of separate high-affinity uptake pathways for  $\text{K}^+$  and  $\text{Na}^+$ , which have been suggested to be mediated by HAK/KUP transporters and HKT transporters, respectively (Garcia-deblas et al., 2003). The rice isoform OsHKT1 is expressed in root epidermal and cortical cells (Golldack et al., 2002), and its kinetic parameters are similar to those displayed by whole roots (high-affinity  $\text{Na}^+$  influx that is blocked by  $\text{K}^+$ ), whereas the isoform OsHKT4 is expressed in shoots and mediates fast  $\text{Na}^+$  influx with low affinity (Garcia-deblas et al., 2003). Recently, a major QTL for shoot  $\text{K}^+$  content and salt tolerance in rice identified the gene *SKC1* encoding an HKT-type protein corresponding to *OsHKT8* (Garcia-deblas et al., 2003; Ren et al., 2005). *SKC1/HKT8* was preferentially expressed in the parenchymal cells surrounding xylem vessels and up-regulated by salt stress (Ren et al., 2005). The *SCK1/HKT8* isoform from the relatively salt-tolerant variety Nona Bokra was more active in the facilitation of  $\text{Na}^+$  transport across the plasma membrane than its counterpart from the salt-sensitive Koshihiraki variety, which suggests that a greater capacity for  $\text{Na}^+$  retrieval from the xylem by *SCK1/HKT8* in Nona Bokra plants was the basis for their salt-tolerance. Together, these results are consistent with facilitated  $\text{Na}^+$  transport in saline conditions, particularly at low  $\text{K}^+$ , and support the concept that ion transport systems and their capacity for  $\text{Na}^+:\text{K}^+$  selectivity are of physiological relevance for the salt tolerance of rice. By extension they also imply that mechanisms restricting net  $\text{Na}^+$  content, including active efflux at the plasma membrane to counteract ion loading, would be determinants for salt tolerance. A precise assessment of the relative importance of SOS1 in the salt tolerance of rice awaits the availability of knock-out mutants or RNA-interference lines.

## MATERIALS AND METHODS

### Isolation of rice cDNAs

An EST clone (Accession C71771) encoding a putative SOS1 homologue in *O. sativa* cv Nipponbare was obtained from the MAFF DNA Bank (Japan). The deduced open reading frame (ORF) was apparently interrupted by two unprocessed introns of 805 and 160 bp at nucleotide positions +750 and +970, respectively (numbering relative to the ATG codon in the spliced cDNA). To obtain a fully spliced cDNA, an RT-PCR was performed with rice RNA as template and the primers: RSOS1A: 5'- GCGTCGACAATCCATGGACAATCCCGAGGCGG, and RSOS1B: 5'- TGTGATAAAATTGGATCCAATGAATGCC, which annealed at the putative initiation codon (underlined) and next to an internal *Bam*HI site, respectively. The RT-PCR reaction yielded a fragment containing a fragment of the ORF, from nucleotides +1 to +2432, that was subcloned in the vector pCR-BluntII-TOPO (Invitrogen Inc.), and fully sequenced to confirm the continuity of the ORF and the fidelity of the amplification. Finally, the amplified fragment was re-introduced in the original EST clone as a *Sall*-*Bam*HI fragment, replacing the equivalent portion in the original cDNA clone with unprocessed introns. The sequence of the corrected cDNA has been deposited in the GeneBank with Accession AY785147.

A putative full-length cDNA clone (AK101368) corresponding to OsCBL4 (according to the nomenclature of Kolukisaoglu et al., 2004) guided the design of oligonucleotides OsCBL4F: 5'-TCGCCATGGGATGCGCGTCGT-3' and OsCBL4R: 5'-TATTTTCAGTCATGGGCTTCT-3' used for the amplification of the open reading frame of OsCBL4 by RT-PCR with total mRNA from *O. sativa* cv Nipponbare as template. The amplified cDNA was fully sequenced to assess the absence of errors. Likewise, the full-length cDNA clone AK102270 encoding OsCIPK24 was used to design the oligonucleotides OsK24F: 5'-GGCGGATGGGAGGGGAGGAGG-3' and OsK24R: 5'-CCAGGCTAGCATGTGGCTGTC-3' for the amplification of the OsCIPK24 by RT-PCR. Amplification from the second methionine at position +7 was done with primer OsK24M2F: 5'-CGCGGATCCGCGGATGGCGGCGGGAGGA-3'. Truncation of the C-terminal part of OsCIPK24 at the leucine residue at position 315/309 was achieved by PCR with an oligonucleotide of sequence 5'-CACCTAAAGAGGGCCACCATC-3', which introduced a stop codon, and primers OsK24F and OsK24M2F, respectively.

#### Plasmid constructs

Plasmid pDROsSOS1 for expression of OsSOS1 in yeast cells under the control of the *PMA1* gene promoter was constructed by subcloning the OsSOS1 cDNA as a 3.7 Kb *Sall*-*NotI* fragment in *XhoI*-*NotI* restriction sites of multicopy vector pDR195 (Rentsch et al., 1996). A C-terminal histidine-tagged version of OsSOS1 for protein purification was created by PCR using the high-fidelity *Pfu* polymerase (Promega) and the primers 5'-AAATGGCAACACATGAGCTCAGGG-3' and 5'-TGAGCGGCCGCTCAGTGATGGTGTGATGTCGATCAGCAGCGCT-3'. The OsSOS1:His6X cDNA was subcloned as a *Bgl*II-*NotI* fragment into pDROsSOS1 to produce



plasmid pDROsSOS1H. A histidine-tagged version of wild-type AtSOS2 was produced by PCR using the primers: 5'-AGAAGCTTATGACAAAGAAAA-3' and 5'-GAGCGGCCGCAAACGTGATTG-3'. The PCR product was digested with *Hind*III and *Not*I and subcloned in the yeast expression vector pYESHis, a derivative of pYES2 (Invitrogen Inc.) modified to contain the epitope RGS6x (Venema et al., 2002). The OsCBL4 cDNA was cloned in pDR195 as a *Xho*I-*Bam*HI fragment, to create plasmid pDROsCBL4. All versions of the OsCIPK24 cDNA (full-length or bearing N- and C-terminal truncations) were cloned as *Eco*RI-*Eco*RI fragments in the yeast expression vector p414GPD (Mumberg et al., 1995). Plasmids pFL2T, pFL3T and pFL32T used for the expression in yeast of *Arabidopsis* SOS2, SOS3 and SOS2/SOS3 proteins, respectively, and plasmid pSOS1-1 for the expression of AtSOS1, have been described elsewhere (Quintero et al., 2002). The plant expression vector pBIOsSOS1 was obtained by two-step cloning of OsSOS1 fragments. First a 2,3Kb *Sal*I-*Bam*HI fragment was inserted into the *Xho*I and *Bam*HI sites of pBI321. Next, a 1,3Kb *Bam*HI-*Bam*HI was inserted in the right orientation to reconstitute a full-length OsSOS1 cDNA. Plasmid pBI321 is a derivative of pBI121 in which the *Xba*I through *Sac*I cloning sites of plasmid pCR2.1 (Invitrogen) were inserted between *Xba*I and *Sac*I sites of pBI121 (Clontech), thus excising the *GUS* coding region of pBI121 and creating additional enzyme restriction sites for cloning. Plasmids pBIOsCIPK24 and pBIOsCBL4 were constructed on the pBI321 backbone for the expression of OsCIPK4 and OsCBL4 in *Arabidopsis*.

#### ***Arabidopsis* transformation and complementation test**

The constructs pBIAtSOS1 (Shi et al., 2000), pBIOsSOS1, pBIOsCIPK24, pBIOsCBL4, and empty vector pBI321 were introduced into the *Agrobacterium* GV3101 strain, and the resulting bacterial clones were used to transform *sos1-1*, *sos2-2* and *sos3-1* *Arabidopsis* mutants by vacuum infiltration (Bechtold et al., 1993). The viral 35S gene promoter is leaky in *Agrobacterium* and the residual expression of SOS1 is deleterious to *Agrobacterium* in the presence of sodium (Martínez-Atienza, unpublished observations). To minimize the selection of re-organized constructs, *Agrobacterium* were grown in LB medium in which 1% KCl substituted for NaCl. Kanamycin-resistant T<sub>2</sub> transgenic plants were selected and subjected to complementation tests on Murashige and Skoog (MS) agar medium supplemented with NaCl as indicated for each case. Culture was in an environmentally controlled chamber at 22°C and 16/8 h daily light/dark cycle with PAR of 30  $\mu\text{mol}/\text{m}^2$  per second.

#### **Rice plants growth conditions**

*O. sativa* cv Nipponbare seeds were germinated in sterile conditions, at 28°C temperature, 100% relative humidity and kept in darkness for five days. Seedlings were then transferred to a hydroponic culture system consisting of boxes containing 8 liters of aerated nutrient solution and holding up to 20 plants each. Nutrient medium consisted of 0.09 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05 mM

$\text{KH}_2\text{PO}_4$ , 0.05 mM  $\text{KNO}_3$ , 0.03 mM  $\text{K}_2\text{SO}_4$ , 0.06 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.07 mM  $\text{MgSO}_4$ , 0.11 mM Fe-EDTA, 4.6  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 1.8  $\mu\text{M}$   $\text{MnSO}_4$ , 0.3  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.3  $\mu\text{M}$   $\text{CuSO}_4$ , pH 5-5.6 (Miyamoto et al., 2001). The hydroponic containers were placed in a growth chamber set to a light/dark cycle of 16/8 h daily (PAR 300  $\mu\text{mol}/\text{m}^2$  per second), 25-20°C day/night temperature, and 60-40% relative humidity. Nutrient solution was changed every seven days. After three weeks of growth in hydroponic medium, rice plants were subject to salt stress by adding NaCl to 100 mM final concentration. Samples of roots and shoots were collected at 0, 3, 15, and 48 h after treatment onset and then frozen in liquid nitrogen.

#### Ribonucleic acid isolation and northern blot

Total RNA was isolated from roots and shoots of salt-treated rice plants and 40  $\mu\text{g}$  from each sample was resolved on 1.25% (w/v) agarose-formaldehyde gel, and transferred to Hybond-N nylon membranes (Amersham) according to standard methods (Sambrook et al., 1989). Radioactive ( $^{32}\text{P}$ ) probe was prepared from a 1.7 Kb *Hind*III fragment of *OsSOS1* C-terminal by use of a random priming kit (Amersham). Probe hybridization was performed overnight at 65°C in a buffer containing 7% (w/v) SDS, 0.5 M  $\text{Na}_2\text{HPO}_4$ , 1% (w/v) BSA, 1 mM EDTA, pH 7.2. The final wash of filters was at 65°C in 0.1X SSC with 0.1% (w/v) SDS. To estimate the relative abundance of *OsSOS1* mRNA in various samples, the RNA loading was normalized through hybridization with the *Eco*RI fragment G of radish ribosomal 18S RNA gene (Delcasso-Tremousaygue et al., 1988).

#### Yeast strains and media

*Saccharomyces cerevisiae* AXT3K strain ( $\Delta\text{ena1}::\text{HIS3}::\text{ena4}$ ,  $\Delta\text{nha1}::\text{LEU2}$ ,  $\Delta\text{nhx1}::\text{KanMX4}$ ) (Quintero et al., 2002) is a derivative of W303-1B (*MAT $\alpha$  ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*). Strain YP890 was derived from AXT3K by chromosomal integration of a *PGK1:SOS1:CYC1* expression cassette into the 3'-end of the *CYC1* gene (Guo et al., 2004). Strain YP1021, carrying a chromosomal integration of a rice *OsSOS1* expression cassette (*PMA1:OsSOS1:ADH1*) was constructed by removing a 1.3 kb *Apal-Sna*BI fragment spanning the *URA3* marker and the 2 $\mu$  DNA replicon from plasmid pDROsSOS1. The resulting plasmid was linearized at the *PMA1* promoter with *Kpn*I and transformed into strain AXT3K carrying plasmid pFL32T. Plasmid pFL32T expresses the SOS2/SOS3 kinase complex and integrative transformants were selected on AP medium supplemented with 200 mM NaCl based on the gain-of-function of SOS1 activity from the *PMA1:OsSOS1:ADH1* cassette thought its activation by SOS2/SOS3. Subsequently, plasmid pFL32T was segregated out in rich, non-selective medium. Yeast transformation was done by the polyethyleneglycol-lithium acetate method (Elble, 1992) and transformants were selected on solid SD drop-out media, except when indicated otherwise.  $\text{Na}^+$  tolerance tests were performed in the alkali cation-free medium AP (Rodríguez-Navarro and Ramos, 1984) supplemented with 1 mM KCl and with NaCl as

indicated for each experiment. For ion content measurements, yeast cells were collected during exponential growth ( $OD_{550} = 0.2$ ) in liquid AP medium, and their  $Na^+$  content was determined by atomic emission spectrometry after acidic extraction (Rodríguez-Navarro and Ramos, 1984).

#### **OsSOS1:His6X purification and phosphorylation assays**

Yeast extracts of cells expressing His-tagged OsSOS1 and AtSOS2 proteins were obtained as described (Serrano, 1988). AtSOS2:His6x was purified from the soluble fraction by chromatography on 1 ml column with  $Ni^{2+}$ -NTA resin (Qiagen) without further processing. To purify OsSOS1:His6x, membrane proteins were purified, solubilized and chromatographed on the  $Ni^{2+}$ -NTA resin as described elsewhere (Quintero et al., 2002). Construction and purification of a glutathione-S-transferase (GST):SOS2T/D $\Delta$ 308 translational fusion has been described also (Guo et al., 2001). Purified OsSOS1:His6x (100 ng) was used as substrate for phosphorylation by the *Arabidopsis* SOS2 and SOS2T/D $\Delta$ 308 protein kinases (100 ng) in 50  $\mu$ l of kinase buffer (20 mM Tris-HCl, pH 8.0, 5 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 1 mM DTT). Reactions were started by adding ATP (0.2 mM with 1  $\mu$ Ci of [ $\gamma$ - $^{32}P$ ] ATP), incubated at 30°C for 30 min, and stopped with 15  $\mu$ l of 4x SDS/PAGE sample buffer. Aliquots were then resolved by SDS/PAGE and the gel exposed to X-ray films.

#### **Purification of plasma membrane vesicles**

Plasma membrane was isolated from yeast cells by the aqueous two-phase system (Menendez et al. 1995). Yeast cells were first cultivated in 200 ml AP (1K) medium containing selection amino acids at 30 °C one day with shaking (200 rpm), and then transferred into 1000 ml fresh medium and cultured at 30 °C with shaking (200 rpm) until the culture reached an  $OD_{600}$  of 3-4. To ensure activation of the SOS2/SOS3 kinase complex, NaCl was then added to final concentration of 100 mM and incubation continued for 1 h before harvesting. Cells were resuspended in 5 mM Tris-HCl, pH 7.5, 700 mM sorbitol to an absorbance of 6 at 800 nm. The volume of the suspension was then measured and mixed with 1/4 of the volume with lyticase (Sigma-Aldrich) dissolved in the above medium at 300 units/ml. DTT was then added in to 6.5 mM final concentration, and the mixture incubated at 30 °C for 1 h with gentle shaking (100 rpm). After lyticase treatment, the resulting protoplasts were recovered by centrifugation at 3000 g for 5 min, washed with the above medium containing 1 mM DTT, and centrifuged again. The pellet was resuspended in 15 ml ATPase-inducing medium containing 15 mM MES-Tris, pH 6.5, 500 mM sorbitol, 100 mM glucose, and incubated at 30 °C for 10 min with gentle shaking (100 rpm). Osmotic lysis was thereafter induced with 30 ml osmotic lysis medium containing 25 mM MES-Tris pH6.5, 5 mM EDTA, 1 mM DTT, 0.2% casein hydrolysate, 0.2% BSA and 1mM PMSF, on ice for 5 min, assisted by a glass homogenizer, and then centrifuged to remove cell wall fragments at 300 g and 4 °C for 3 min. The supernatant was collected and centrifuged at 35000 g and 4 °C for 15 min. The pellet was resuspended in 9 g membrane suspension medium

containing 5 mM potassium phosphate pH 7.8, 330 mM sucrose and 1 mM DTT, and mixed quickly with 27 g of two-phase solution with the final composition of 5.7% dextran T-500 (w/w), 5.7% PEG 3350 (w/w), 5 mM potassium phosphate pH 7.8, 330 mM sucrose, 1 mM EDTA and 1 mM DTT. The two-phase system was settled on ice for 30 min until phases separated. The upper and lower phases were collected separately, diluted with 10-fold the fraction volume of 15 mM MES-Tris pH 6.5, 330 mM sucrose, 1 mM DTT, and centrifuged at 65000 g and 4 °C for 50 min. The membranes were resuspended in the same medium and frozen in liquid nitrogen and stored at -70 °C until use.

#### **Na<sup>+</sup>/H<sup>+</sup> exchange assays**

The formation of  $\Delta$ pH was established by the activity of the plasma membrane H<sup>+</sup>-ATPase and Na<sup>+</sup>/H<sup>+</sup> exchange activity was measured as a Na<sup>+</sup>-induced dissipation of  $\Delta$ pH with the pH value sensitive fluorescent probe quinacrine in the following reaction mixture (1 ml): 5  $\mu$ M quinacrine, 50 mM BTP-HCl pH 7.5, 25 mM BTP-Hepes pH 7.5, 250 mM mannitol, 4 mM MgSO<sub>4</sub> and 50  $\mu$ g plasma membrane protein. The reaction mixture was placed in a fluorescence spectrophotometer (Hitachi F-2500) and equilibrated in the dark with stirring for 5 min before fluorescence measurement. The assay was initiated with the addition of 3 mM ATP. When  $\Delta$ pH reached steady state, equal amounts of various concentrations of Na<sub>2</sub>SO<sub>4</sub> or sodium gluconate stock solutions were added to the reaction mixture. To determine initial rates of Na<sup>+</sup>/H<sup>+</sup> exchange, the change of relative fluorescence was measured during the first 30 seconds after the addition of sodium salts. Specific activity was calculated by dividing the initial rate of fluorescence recovery, expressed as a ratio of the preformed pH gradient, by the mass of plasma membrane protein in the reaction and time ( $\Delta F \text{ mg}^{-1} \text{ min}^{-1}$ , where  $\Delta F = F_{30} - F_0 / F_{\text{max}} - F_{\text{min}}$ ). The change of pH value was measured at excitation and emission wavelengths of 430 and 500 nm respectively.

#### **Distribution of materials**

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY785147.

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## FIGURE LEGENDS.

**Figure 1. Activation of rice SOS1 by the *Arabidopsis* SOS2/SOS3 kinase complex. (A)** AXT3K cells transformed with an empty vector (0) or with the indicated combination of SOS genes (1, OsSOS1; 2, AtSOS2; 3, AtSOS3) were grown overnight in selective SD medium. Five microliters of serial decimal dilutions were spotted onto plates of AP medium with 1 mM KCl and supplemented with 0 (not shown), 100 or 200 mM NaCl. Plates were incubated at 28°C for 3 days. Plasmids used for expression of the SOS proteins were pDR195 for OsSOS1, pFL2T for AtSOS2, pFL3T for AtSOS3, and pFL32T for co-expression of AtSOS2 and AtSOS3. **(B)** Intracellular Na<sup>+</sup> content as determined by atomic emission spectrometry. Cells were grown in AP medium with 1 mM KCl and 30 mM NaCl, and collected at OD<sub>550</sub>=0.2-0.3. Values are the mean and SE of 3 independent cultures of each combination of SOS genes. Units are nanomoles of ion per milligram dry weight of cell samples.

**Figure 2. Na<sup>+</sup>/H<sup>+</sup> antiporter activity of rice SOS1. (A)** ATP-dependent pH gradient formation in membrane vesicles isolated from yeast cells expressing rice SOS1. An inside-acid ΔpH was formed after the addition of ATP to vesicles (arrow 1). Once fluorescence was stabilized, sodium salts were added to the cuvette (arrow 2), and fluorescence recovery, indicating proton exchange, was monitored for 2 minutes, after which ΔpH was disrupted by the addition of 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (arrow 3). Fluorescence is expressed as arbitrary units. **(B)** Na<sup>+</sup>/H<sup>+</sup> exchange as a function of Na<sub>2</sub>SO<sub>4</sub> concentration and the presence of rice OsSOS1, with and without co-expression of the *Arabidopsis* SOS2/SOS3 kinase complex. Circles, AXT3K cells transformed with empty vector pDR195; squares, AXT3K cells expressing rice OsSOS1 alone; diamonds, AXT3K cells transformed to produce proteins OsSOS1, AtSOS2 and AtSOS3. Na<sup>+</sup>/H<sup>+</sup> exchange activity is given as the proportion of dissipation of the preformed pH gradient per minute and milligram of membrane protein. **(C)** Specificity of sodium-induced proton exchange. Sodium was added to a final concentration of 75 mM as sulfate (grey bars) or gluconate (black bars) salt. Values are the mean and SE of percent fluorescence dissipation of triplicate samples.

**Figure 3. Phosphorylation of rice SOS1 by the *Arabidopsis* kinase SOS2. (A)** Recombinant His-tagged OsSOS1, AtSOS2 and GST-fused AtSOS2T/DΔ308 were purified by affinity-chromatography. Aliquots (1 to 4) of the first elution volumes were analyzed for protein purity by SDS-PAGE. Bands corresponding to OsSOS1:His6x (128 kDa), AtSOS2:H6x (52 kDa) and GST:AtSOS2T/DΔ308 (60 kDa) are indicated. Standard molecular weights are shown on the left. **(B)** Purified proteins were combined as indicated in protein kinase reaction assays. Aliquots of phosphorylation reactions were resolved by SDS-PAGE and exposed to X-ray films. Arrow indicates the 128 kDa band pertaining to phosphorylated OsSOS1.

**Figure 4. Complementation of *Arabidopsis* sos1-1 mutant by rice OsSOS1. (A)** Six-day old seedlings grown on MS agar medium were transferred to MS medium supplemented with 50

mM NaCl and imaged after 14 days of salt treatment. Left, transgenic *sos1-1* mutant seedling expressing AtSOS1 from the cauliflower mosaic virus 35S promoter. Center, 5 independent transgenic lines of *sos1-1* mutants expressing the rice OsSOS1 gene under the control of the 35S promoter. Right, a *sos1-1* mutant seedling transformed with empty vector pBI321. (B) Quantitation of seedling growth, expressed as fresh weight after 14 days in MS medium with and without supplemental 65 mM NaCl. Data are the mean and SE of fresh weight values of 3-6 individual seedlings from each line. Dashed bars, *sos1-1* mutant seedlings transformed with empty vector pBI321; grey bars, *sos1-1* mutant seedlings transformed with the rice OsSOS1 gene under the control of the 35S promoter; black bars, *sos1-1* mutant seedlings transformed with *Arabidopsis* SOS1.

**Figure 5. Transcript abundance of OsSOS1 in response to salt stress.** Total RNA purified from root and shoot of rice plants subjected to salt stress with 100 mM NaCl in hydroponic culture medium for 0, 3, 15, and 48 hours. Hybridization was performed with a probe prepared from OsSOS1 cDNA. RNA sample loading was normalized by hybridization with a probe derived from radish 18S rDNA.

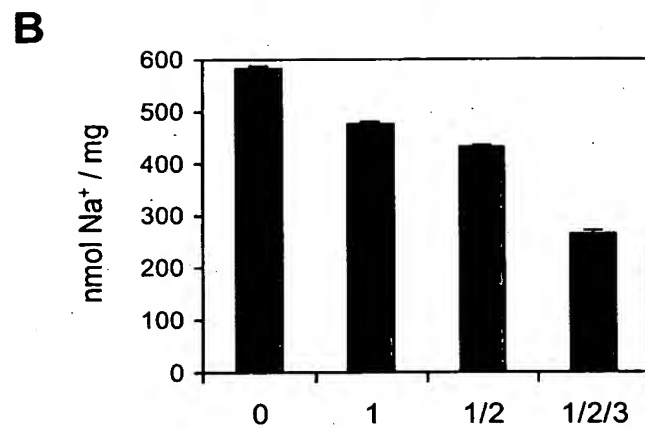
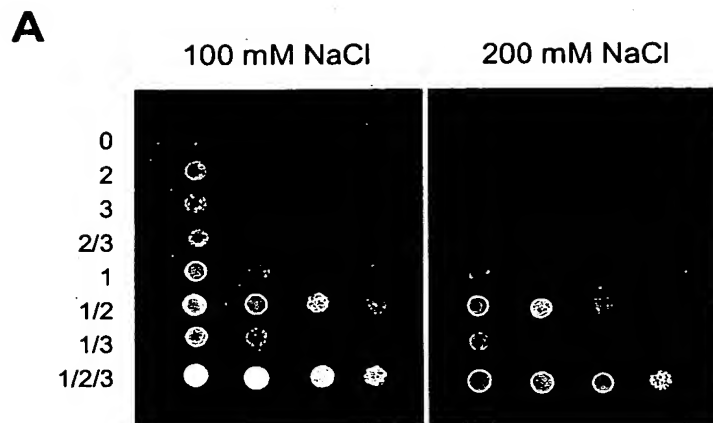
**Figure 6. Functional interactions between Arabidopsis SOS proteins and rice counterparts.** Strains YP890 carrying the integration of the *PGK1<sub>PRO</sub>:AtSOS1:CYC1<sub>TER</sub>* cassette (A, C), YP1021 with the analogous integration *PMA1<sub>PRO</sub>:OsSOS1:ADH1<sub>TER</sub>* with the SOS1 cDNA from rice (D), and AXT3K cells transformed with plasmid pSOS1-1 for the expression of AtSOS1 (B) were transformed with plasmids directing the expression of the regulatory proteins SOS2 and SOS3 from *Arabidopsis* or CIPK24 and CBL4 from rice, as indicated in each case. CIPK24 $\Delta$ 309 bears a C-terminal deletion rendering a constitutive, CBL-independent protein kinase. Yeast cells were grown overnight in selective SD medium. Five microliters of serial decimal dilutions were spotted onto plates of AP medium with 1 mM KCl and 200 mM NaCl. Plates were incubated at 28°C for 3-4 days.

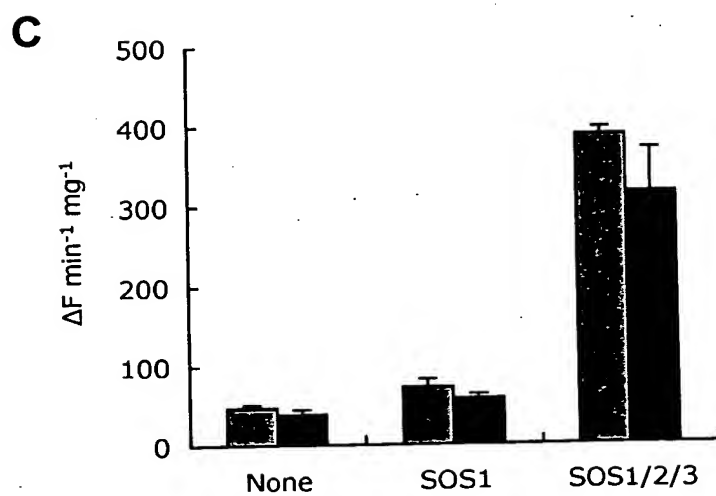
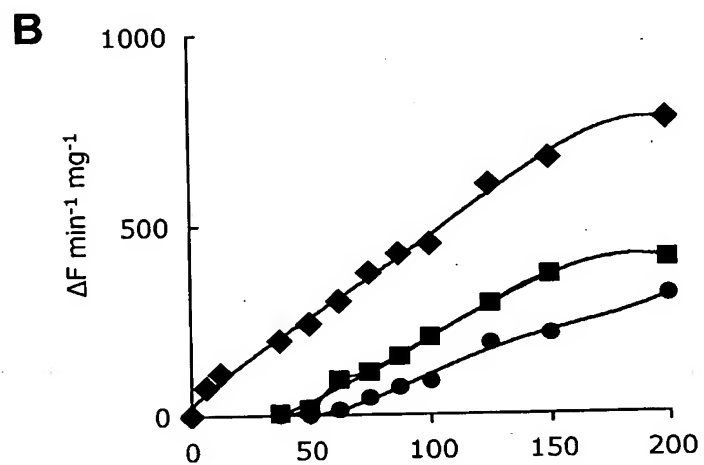
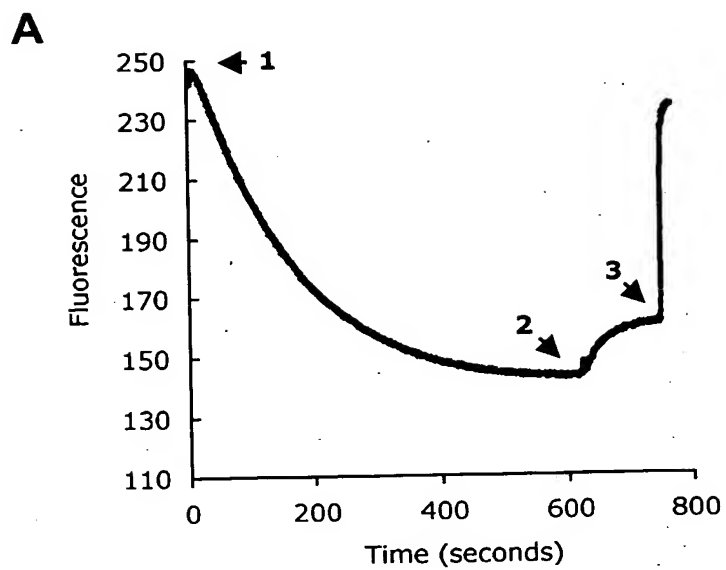
**Figure 7. Complementation of Arabidopsis sos2 and sos3 mutants by rice CIPK24 and CBL4.** Six-day old seedlings of mutants *sos3-1* (A) and *sos2-2* (B) and transformed with cDNAs of rice genes CBL4 (A) or CIPK24 (B) respectively, were transferred to MS medium supplemented with 100 mM (CBL4 and *sos3*) or 75 mM NaCl (CIPK24 and *sos2*) and imaged after 14 days of growth. In both panels, from left and right, two wild-type plants, five complemented lines, and two mutant plants transformed with empty vectors are depicted. The root length of seven individual plants from each of these lines was measured after 14 days in salinized media (C, *sos3* mutant and rice CBL4; D, *sos2* mutant and rice CIPK24). In both plots, dashed columns represent the root length attained by the mutant lines, gray columns are the complemented lines, and back columns are the wild-type Col-0.

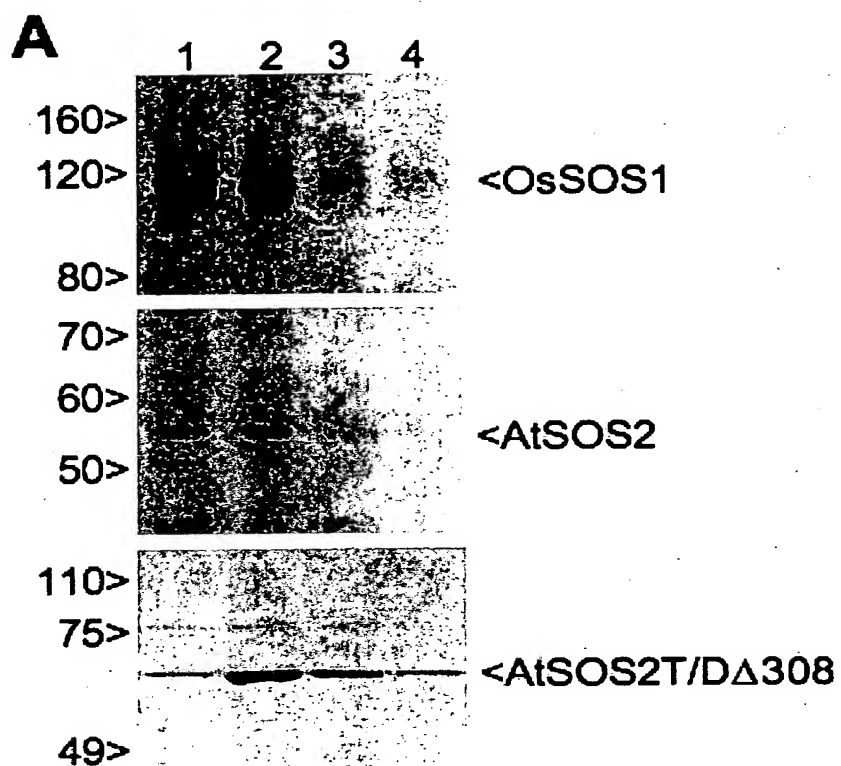
**Supplemental Figure 1. Phylogenetic relationships of SOS1 homologues from various plant species.** (A) Amino acid sequence alignment of SOS1 proteins from *Arabidopsis* (top sequence) and rice (bottom sequence). Alignment analysis was performed with CLUSTAL-X (Thompson et al., 1997). Identical residues are highlighted in black. The predicted 12 transmembrane (TM1-TM12) segments are indicated. The position of 2 unprocessed introns removed from OsSOS1 cDNA are indicated by asterisks. (B) Putative polypeptides homologous to *A. thaliana* SOS1 were aligned by use of CLUSTAL-X. Illustrated is the phylogenetic tree of proteins from *A. thaliana* (Accession E84431), *O. sativa* (Acc. C71771), *Physcomitrella patens* (Acc. CAD91921), *Cymodocea nodosa* (Acc. CAD20320), together with these from *Thellungiella halophila* and *Mesembryanthemum crystallinum* (Valery Poroyko and Hans Bohnert, University of Illinois; personal communication).

**Supplemental Figure 2. Amino acid sequence alignment of AtSOS3 from *A. thaliana* and OsCBL4, 7 and 8 from *O. sativa*.** Identical residues in all the proteins are highlighted in black; residues conserved relative to AtSOS3 but not present in all isoforms are shaded in gray. The double line indicates the consensus sequence for N-myristoylation (MGXXXS) and the arrowhead the putative glycine residue to which myristate is attached. Segments corresponding to the putative EF-hand domains of this  $\text{Ca}^{2+}$ -binding proteins are indicated as EF1 to EF4.

**Supplemental Figure 3. Amino acid sequence alignment of AtSOS2 from *A. thaliana* and OsCIPK24 from *O. sativa*.** Identical residues are highlighted. Grey arrowheads indicate the 2 alternative methionine residues (M1 and M7) used for the translational initiation of the OsCIPK24 cDNA. Only the shorter protein starting at M7 was active. The NAF/FISL domain for binding to CBL proteins is underlined. The black arrowhead indicates the C-terminal truncation (leucine at position 308 in AtSOS2; position 309 in OsCIPK24, relative to methionine at position 7) that results in a constitutively active, CBL-independent protein kinase.



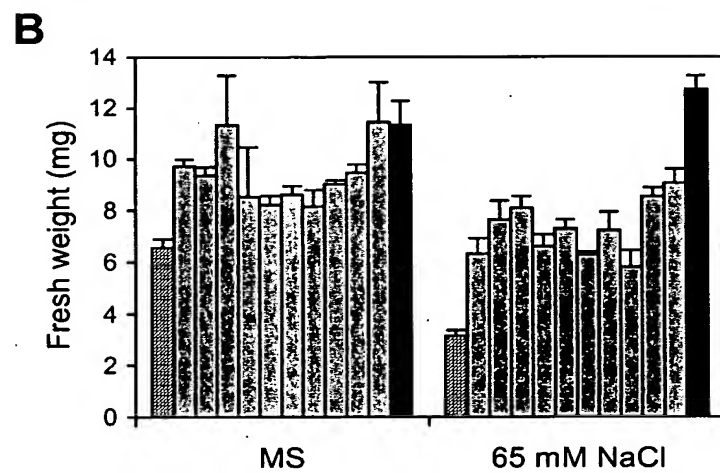
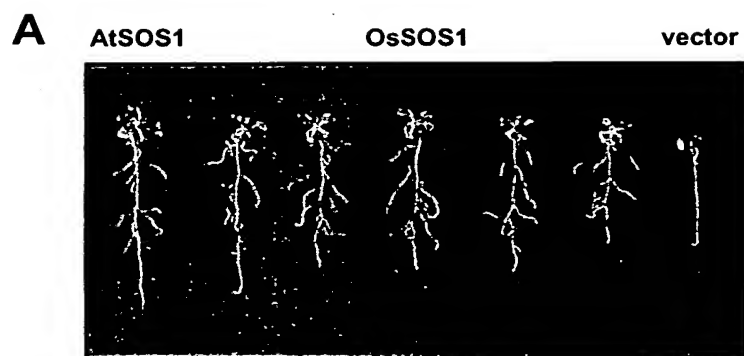


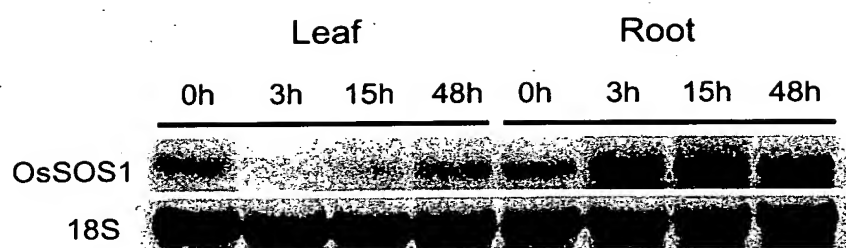


**B**

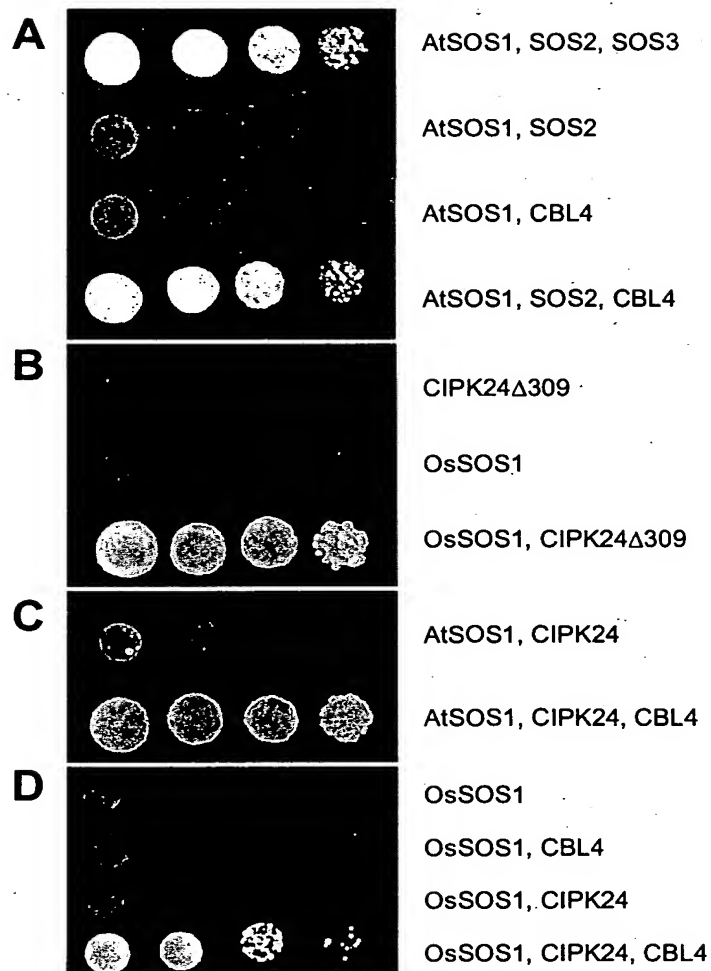
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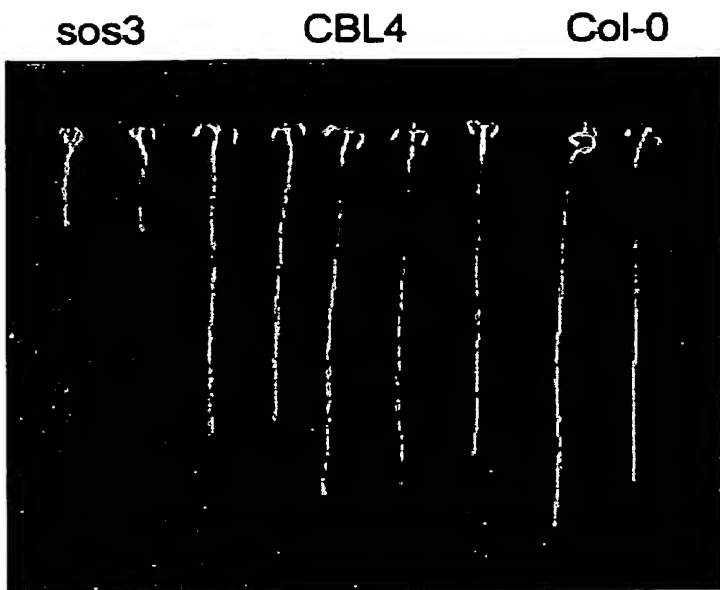
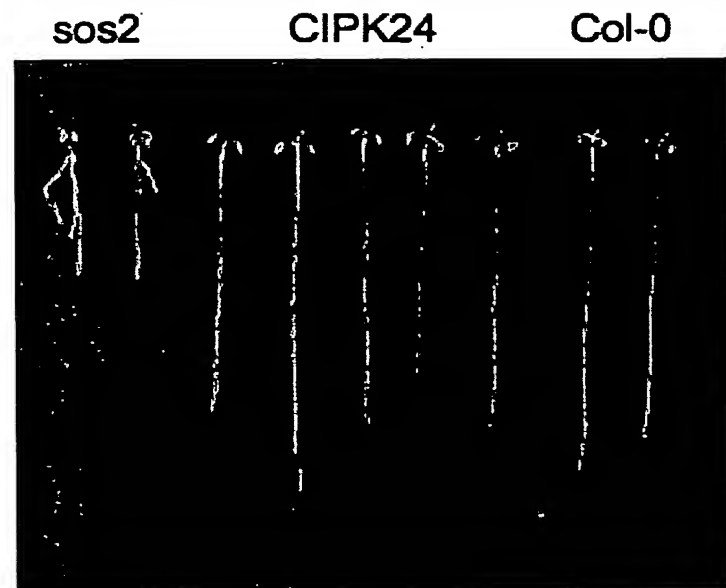
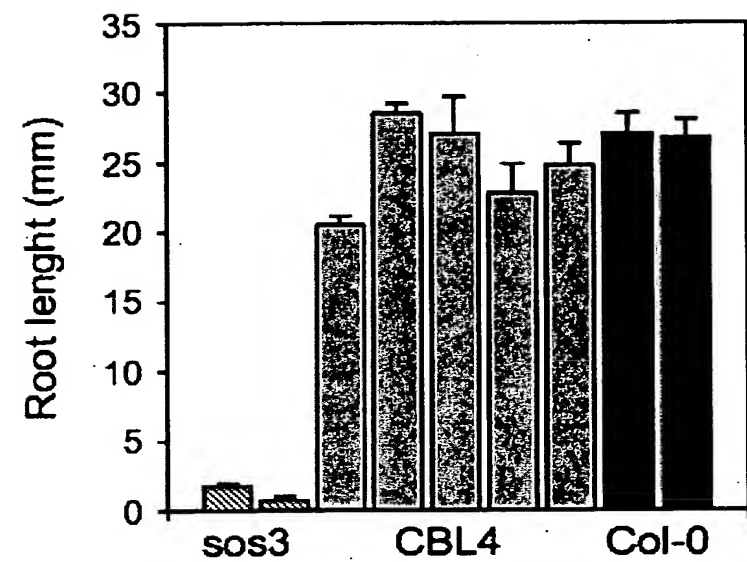










**A****B****C****D**